DEPARTMENT OF HEALTH AND HUMAN SERVICES

NOTE TO THE FILE
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Subject: Insect Resistant and Glufosinate Tolerant Corn Line CBH-351

Keywords: corn, *Zea mays*, lepidopteran insect resistance, European corn borer, Ostrinia nubilalis, *Bacillus thuringiensis* subsp. *tolworthi* (*Bt tolworthi*) *cry9C* gene, insect control protein Cry9C, glufosinate, herbicide tolerant, *Streptomyces hygroscopicus bar* gene, phosphinothricin acetyltransferase (PAT).

Background

AgrEvo USA Company, along with its recently acquired subsidiary, Plant Genetic Systems, initiated consultation with FDA regarding this product in November of 1996. On March 3, 1998, AgrEvo provided summary information to support its safety and nutritional assessment of a new insect resistant and herbicide tolerant corn line containing transformation event CBH-351.

Intended Effect and Food/Feed Use

The intended effect of this genetic modification of corn is to confer resistance to lepidopteran larvae, including the European corn borer, and tolerance to the herbicide glufosinate ammonium. AgrEvo used the *cry9C* gene from *Bacillus thuringiensis* subsp. *tolworthi* (*Bt tolworthi*) to confer insect resistance in the CBH-351 corn line. AgrEvo also integrated the *bar* gene derived from *Streptomyces hygroscopicus* which encodes phosphinothricin acetyltransferase (PAT) into the CBH-351 corn line. PAT confers resistance to the herbicide phosphinothricin, also known as glufosinate. According to AgrEvo, the integration of the *bar* gene enables the selection of the pest resistant line independent of the plant stage and provides tolerance to glufosinate-ammonium herbicides.

Corn is one of the world's primary cereal grain crops. Corn grain (kernels) has both animal feed and human food applications. Human food use of the grain includes direct consumption of the kernels, which constitutes 2-3% of total domestic corn consumption, and the production of high fructose corn syrup, glucose, corn oil, starch, ethanol and corn meal, which in total constitutes about 20% of total domestic corn consumption. More than 75% of domestically produced corn grain is used in animal feed, primarily for poultry, swine, and cattle. Animal feed uses for corn include: 1) the grain, which include the kernel itself, fed whole or in a processed form; 2) the byproducts from the production of human food; and 3) silage which is produced from the entire corn plant.

Molecular Alterations and Characterization

AgrEvo used microprojectile bombardment to introduce two plasmids simultaneously into hybrid corn tissue derived from the public inbred line, H99. According to AgrEvo, line H99 has superior

qualities in tissue culture, particularly its high frequency of type I callus formation. One plasmid contained a modified version of the *Bt tolworthi cry9C* gene, *cry9C.PGS2a*. AgrEvo used a Cauliflower Mosaic Virus (CaMV) 35S promoter and a Petunia *cab22L* leader sequence to direct high level constitutive expression of the *cry9C.PGS2a* gene. The *cry9C.PGS2a* gene has three modifications from the wild type *cry9C* gene. When expressed in the transformed plant the *cry9C.PGS2a* gene produces a Cry9C protoxin that is: 1) truncated at the C-terminal end at amino acid position 666; 2) truncated at the N-terminal end at position 43 with two amino acids, methionine and alanine, added to the truncated N-terminus; and 3) comprised of lysine instead of arginine at position 123. According to AgrEvo, the replacement of arginine by lysine reduces the susceptibility of the protein to trypsin cleavage. The insecticidal activity of the Cry9C protein is unaffected by these changes.

The second plasmid contains the *bar* gene derived from *Streptomyces hygroscopicus* under control of the CaMV 35S promoter. The *bar* gene encodes the PAT protein. PAT acetylates glufosinate ammonium, the active ingredient of a variety of commercial herbicides, and thereby, detoxifies the herbicide. Both plasmids contain an origin of replication (ori) required for replication of the plasmids in *E. coli*, and the *β-lactamase* gene (*bla*) which confers resistance to antibiotics, such as ampicillin, in bacteria. AgrEvo reports that the *bla* gene is under control of a bacterial promoter that functions only in procaryotes.

To characterize the DNA insertion in transgenic plant line CBH-351, AgrEvo carried out molecular analyses. AgrEvo performed Southern blot analyses to determine the presence or absence of an inserted gene in the transgenic plant and the number of copies of the transgene. The results presented in their safety assessment indicate that, in CBH-351 plants, there are four copies of the *bar* gene, one copy of the *cry9C.PGS2a* gene, and five copies of the *bla* gene all inserted at a single site in the corn genome. The presence of the *bla* gene was verified by AgrEvo through use of the polymerase chain reaction (PCR).

AgrEvo assessed the stability of the inserted DNA in the CBH-351 corn line by Southern blot analyses, and segregation and linkage analyses. Using Southern blot analyses, AgrEvo examined the primary transformant and five generations of offspring using a probe that contains DNA used in the original transformation event. The results presented in AgrEvo's safety assessment show an identical Southern blot pattern for all analyzed offspring and the primary transformant. According to AgrEvo, these results imply that the inserted DNA in corn line CBH-351 is stably inherited across generations. From segregation analyses, AgrEvo determined that the herbicide tolerance and insect resistance traits in the transgenic corn line CBH-351 are closely linked at a single locus in the genome.

Expressed Proteins

AgrEvo tested for the expression of the inserted genes by Northern blot analyses which can detect the presence of mRNA and by enzyme linked immunosorbent assay (ELISA) which can detect the presence of protein. Expression of the *cry9C.PGSa* gene and the *bar* gene was observed by

Northern blot analyses in leaf, stem, root, ear and tassel but not in seed. For the *bla* gene, no positive signal could be detected in leaf, root, stem, or seed by Northern blot analyses. AgrEvo concludes that the *bla* gene present in corn containing transformation event CBH-351 is not expressed in the plant tissues.

AgrEvo determined the amount of Cry9C and PAT protein in different tissues and at different stages of plant growth by ELISA analyses, and reports that both proteins represent only a small portion of the total plant protein. In plants grown until ready for harvest as silage (silage stage), Cry9C makes up 1.7% and 0.4% of total protein for whole plant tissue and leaf tissue, respectively. In plants grown until ready to harvest for grain (harvest stage), Cry9C makes up 0.9%, 1.3%, and 0.3% of total protein for whole plant tissue, leaf tissue and kernel, respectively. PAT represents 0.8%, 0.2%, and 0.3% of the total protein for whole plant tissue at the silage stage, leaf tissue at the silage stage and whole plant tissue at the harvest stage, respectively. PAT protein levels in leaf tissue and kernel at the harvest stage are below the detection limit of the assay, 1.5 µg PAT/g dry weight.

In addition to the whole kernel, AgrEvo determined the amount of Cry9C and PAT protein in processed grain fractions by ELISA. Measured as a percentage of the total soluble protein for each of the processed fractions tested, PAT and Cry9C individually contribute to less than 1% of the total soluble protein, and in the crude or refined oil, neither protein was detected.

Regulatory Considerations

The safe use of the insecticidal protein, Cry9C, as a pesticide is regulated by the Environmental Protection Agency (EPA) under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA). EPA regulates the use of the introduced genetic material encoding the Cry9C protein (including associated sequences required for expression) as well as the expression product. Although, for our information, AgrEvo discussed the molecular characterization of the *cry9C.PGS2a* gene introduced into its corn line CBH-351 and the safety of the Cry9C Bt toxin, the safety issues related to pesticides and the introduced genetic material used to produce pesticides are under the purview of EPA. Therefore, we have not addressed the safe use of Cry9C as a pesticide or the safe use of the *cry9C.PGS2a* gene in the production of transgenic corn.

We have considered, however, the safety of PAT which renders the transgenic corn plant tolerant to the herbicide, glufosinate ammonium, and possible alterations in levels of nutrients and anti-nutrients that may have occurred in corn line CBH-351 as a result of the genomic modification. AgrEvo states that the safety of PAT has been addressed previously. The safety of PAT, encoded by the *bar* gene, was the subject of a consultation (BNF0029) that was completed on December 14, 1995.

Compositional Analysis

Silage

The intent of the genetic modifications made by AgrEvo was to produce insect resistant and herbicide tolerant corn. AgrEvo did not anticipate any other effect from the introduction of the transgenes into corn. To confirm this expectation, AgrEvo performed compositional analyses on silage (green chop) made with non-transgenic hybrid corn plants (untreated control), hybrid corn plants containing event CBH-351 (untreated transgenic corn), and hybrid corn plants containing event CBH-351 sprayed with glufosinate (treated transgenic corn). AgrEvo reports that with a few exceptions, the values from proximate analyses (moisture, fat, protein, ash, carbohydrate, neutral detergent fiber, and acid detergent fiber) for the treated and untreated transgenic corn were within the range of the untreated control. Some differences were found in the values for specific components of the proximate analyses between the treated and untreated transgenic corn at one location, but these differences were not consistent in all repetitions and were within the range when all samples were considered.

AgrEvo presented data from analyses for the anti-nutritional substance, phytic acid, in silage from transgenic corn plants and control corn plants grown under the same conditions as described for the proximate analyses mentioned above (untreated control, untreated transgenic corn, and treated transgenic corn). AgrEvo concluded that although differences were observed between the untreated control and the untreated and treated transgenic corn, all values were below the 0.20% phytic acid content considered to be the lower limit for anti-nutritional effects from phytic acid.

Grain

Again to confirm the expectation that no unintended effect may have rendered the transgenic corn inferior for food or feed consumption, AgrEvo conducted compositional analyses on corn grain from hybrid corn plants containing event CBH-351 (transgenic corn), their non-transgenic hybrid counterparts (non-transgenic corn), and other standard hybrids (standard corn). Grain samples were analyzed by standard methods for moisture, fat, protein, fiber, ash, calcium, phosphorus, and amino acid profile. No statistically significant differences between transgenic and non-transgenic corn in the levels of moisture, fat, ash, calcium and phosphorus are reported by AgrEvo. The values for protein and crude fiber content for the transgenic corn grain differed from those observed for the non-transgenic corn grain. However, these values were similar to or higher than USDA literature data. According to AgrEvo, these results indicate that neither the transgene product nor the transformation process account for the values outside of the reference literature range.

For many of the amino acids, the content of a particular amino acid in the transgenic corn grain is similar to those in the non-transgenic and standard corn, but is higher than the USDA reference value. According to AgrEvo, the higher amino acid values are not surprising since a higher value for total protein was observed. Again, AgrEvo notes that since the non-transgenic and standard corn grains have values for amino acid content similar to the transgenic corn, the results indicate that the presence of foreign DNA into the transgenic corn cannot account for the values being

higher than the reference values.

Conclusions

AgrEvo has concluded that its transgenic corn line containing transformation event CBH-351 is not materially different in terms of food safety and nutritional profile from corn varieties currently on the market. At this time, based on AgrEvo's description of its data and analyses, the Agency considers AgrEvo's consultation on corn lines containing transformation event CBH-351 to be complete.

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